TENDON CREEP IS POTENTIATED BY NKISK AND RELAXIN WHICH PRODUCE COLLAGEN FIBER SLIDING

Mark L Wood, MD; William N Luthin, BS; Gayle E Lester, PhD; Laurence E Dahners, MD

ABSTRACT

The pentapeptide NKISK has been reported to inhibit the binding of decorin, a proteoglycan on the surface of collagen fibrils, to fibronectin, a tissue adhesion molecule. Relaxin has been shown to be effective in relaxing ligaments and other connective tissues. Through collagen staining studies, we have previously demonstrated that collagen fiber sliding is important during changes in ligament length. Because of our interest in fibril-fibril binding as it relates to changes in length of tendon or ligament, we investigated the potential of NKISK, relaxin, or both in combination to potentiate creep. We suspended stained rat tail tendons in test solutions under a constant stress and observed length changes and subsequent collagen fiber sliding. Both NKISK and relaxin potentiated rat tail tendon creep with changes in length most likely occurring as a result of collagen fiber sliding as evidenced by photomicrography.

INTRODUCTION

It is accepted that tendon and ligament have the ability to change lengths[2, 5,9,10,19]; however, little is known about the mechanism responsible for these phenomena. Our laboratory has focused on the hypothesis that sliding of collagen fibers past one another plays an important role during these length changes. We have shown in previous in vivo experiments that ligament length changes occur by the movement of collagen fibers past one another during growth and contracture[20] and that the length changes occur diffusely throughout the entire ligament or tendon[2,5,9,10,19]. The "sliding fibril hypothesis" requires that tapered, discontinuous fibrils[7,14,16,17] have a reversible mechanism of fiber to fiber bonding, which releases to allow such diffuse changes in length. A better understanding of collagen interaction during length changes is important because very little is currently known about the development or the mechanism of wound healing in tendon, ligament and other collagen-rich tissues.

Previous work in our laboratory has examined the impact of modifying interfibrillar bonding by immersing stressed tendons in different solutions (varying in pH, calcium ion concentration, osmolarity and concentrations of acetylcholine and epinephrine) all without success. We have, however, found and reported increased strain in stressed tendons when immersed in gentamycin and polylysine, both of which are small polycations with similar charge configurations[11].

Schmidt, et al showed that a pentapeptide, NKISK, competitively inhibits binding of fibronectin to decorin by mimicking the sequence in the decorin molecule, which is presumed to be the fibronectin binding site[12]. Decorin is a small proteoglycan which "decorates" the surface of collagen fibrils and fibronectin frequently functions as an adhesion molecule. Hedbom and Heinegard have proposed that one possible function of decorin is to connect neighboring collagen fibrils[6]. Unpublished work from our laboratory has shown that stressed tendons immersed in NKISK or in relaxin (a hormone effective in relaxing ligaments and other connective tissues)[1,8,13,18] stretch significantly more than control tendons and we have published that free, intact fibrils can be isolated after exposure of ligament to gentamycin or NKISK[2,4].

This study focuses on the effect of NKISK and/or relaxin as well as the possible additive effect of the two agents (in combination) on interfibrillar bonding and on subsequent collagen fiber sliding in the stressed rat tail tendon model. By applying a covalently bonding, fluorescent collagen dye, we were able to label collagen fibers and observe subsequent movement during tendon strain.

Department of Orthopaedic Surgery
University of North Carolina
Chapel Hill, North Carolina

Address for Correspondence:
Laurence E. Dahners, MD
237 Burnett-Womack Building, CB # 7055
UNC School of Medicine
Chapel Hill, NC 27599-7055
(919) 966-3340
Fax: (919) 966-6730
Email: led@med.unc.edu

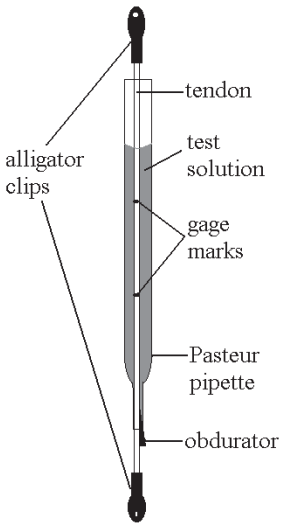

Figure 1. Diagram of the rat tail tendon suspension/loading apparatus.

METHODS

NKISK was synthesized for this experiment by the University of North Carolina Peptide Synthesis Facility and porcine relaxin was provided by Dr. Michael Fields at the University of Florida, Gainesville. The test solutions were 1 m*M* NKISK and 46 units/ml porcine relaxin (3,000 units/mg) in commercially prepared phosphate buffered saline (PBS - Invitrogen Corp, Grand Island, NY - 136m*M* NaCl, 3m*M* KCl, 1 m*M* KH_2PO_4, and 6 m*M* Na_2HPO_4), pH 7.4, to which 0.03% NaN_3 had been added. These concentrations were chosen after they were found to be appropriate in previously performed pilot studies. PBS with 0.03% NaN_3 was the control solution.

After Institutional Review Board approval, rat tail tendons were harvested from sacrificed, fresh frozen, 500 g Sprague Dawley rat cadavers under a dissecting microscope. Each experiment was done with the tendons from a single rat tail, and from the same segment of that tail (2 tails). The tails were thawed for 15-30 minutes then cut through a vertebral disc near the base of the tail and again 6 cm distally. Micro forceps were used to pull tendons out of the distal end of the tail under a dissecting microscope, obtaining similar (medium) sized tendons. The tendons were marked twice, at a gauge length of 15 mm near the center of the tendon segment using a 25 gauge hypodermic needle dipped in India ink. The tendons were pulled individually into glass tubes made from Pasteur pipettes, cut just below the tapered section and again 2 cm above the taper, so that the ends of the tendon protruded from both ends of the pipette segment tubes. The bottom end of the tube was plugged with silicone vacuum grease and a small obdurator, then the tube was filled with the test solution. During the experiment, small volumes of evaporation (approximately 1–2% of the weight of the entire apparatus) were replaced.

Alligator clips grasped the ends of the tendons and the tendon/tube apparatus was suspended from the upper clip (Figure 1). This assembly was weighed at the end of the experiment to determine the load applied to the tendon. There were 4 models (control, NKISK, relaxin, and NKISK/ relaxin mixture) each consisting of 10 tendons (a total of 40 tendons). The distance between the India ink gage marks was blindly measured (twice, then calculating the average) using a 1.5X magnifier and a micro caliper accurate to 0.02 mm at the beginning of the suspension period and again after 3, 6, 12, 27, 36 and 48 hours. The experiment was repeated using the above procedure in a second rat tail with the same 4 models, each consisting of 4 tendons (a total of 16 tendons).

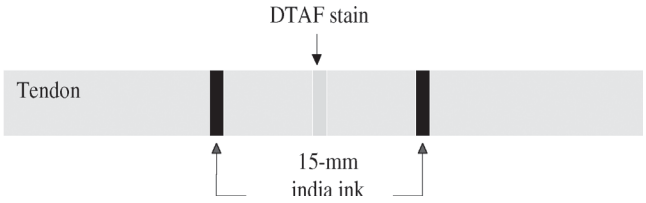

Figure 2. A diagram representing a rat tail tendon and the initial India ink marks with a dichlorotriazinyl fluorescein (DTAF) stain applied perpendicular to the collagen fibers.

In the 2nd experiment, the tendons were stained (perpendicular to the collagen fibers) between the ink marks, with an extremely thin line (Figure 2) using a 10-0 nylon suture dipped in the fluorescent collagen dye dichlorotriazinyl fluorescein (DTAF—5 mg/ml in fresh 0.2 M sodium bicarbonate)[3]. A picture, demonstrating the DTAF stain width, was taken with a fluorescence microscope at 16X magnification. Utilizing photomicrography for measurements allowed standardization between individual specimens as well as blinded measurements.

Each tendon was then suspended for 27 hours at which time a final picture was taken using the fluorescence microscope at 16X magnification. The initial and final fluorescent marking widths were blindly measured (twice, then calculating the average) on the 16X photomicrographs using the caliper (accurate to 0.02 mm) and the percent increase in width (defined as the increase in width normalized to the initial width, expressed as a percent) of the mark was calculated. When the edges of the marks were not sharp, the most uniform area was measured from "tip to tip" in both the initial and final photomicrographs. In both experiments, the India ink mark measurements were used to calculate the percent creep (defined as the increase in length normalized to the initial length, expressed as a percent) at each of the time points. At the end of the experiment stress was calculated[15]. The portion of tendon between the India ink marks was isolated using a scalpel under the dissection microscope and then air dried for three days. Each segment was weighed for 0.00 mg accuracy. For the 15 mm segment, multiplying the weight by the constant 0.133 (this depends on the tendon being 57%

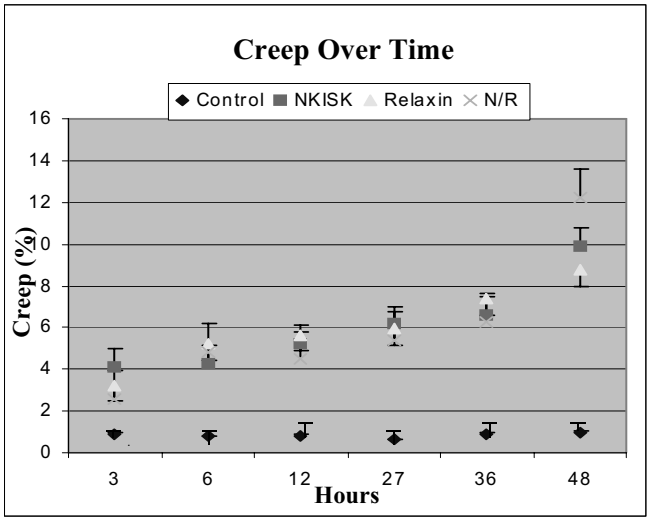

Figure 3. Percent creep over time for rat tail tendons exposed to NKISK, relaxin, and NKISK/relaxin mixture models versus control (PBS). Hash marks represent standard error and all experimental values are significant versus control ($p<0.05$).

water, 43% dry material and having a density of 1.16 gm/cc) converted that weight to cross sectional area in mm^2. Stress is measured in Megapascals (Mpa = N/mm^2), so conversion of the grams of the load to N (9.8N=1 Kg) was done by multiplying by 0.0098.

Stress was calculated by dividing this load by the cross sectional area. Differences between models versus control were evaluated for statistical significance using a Bonferroni modified Student's t-test.

RESULTS

The first experiment consists of 40 tendons (10 per model) without photomicrographs and the second experiment 16 tendons (4 per model) with photomicrographs. In the first experiment, the mean tendon stress in the control, NKISK, relaxin, and NKISK/relaxin mixture models were 0.662 +/- 0.116 (SE) MPa, 0.383 +/- 0.079 (SE) MPa, 0.589 +/- 0.102 (SE) MPa, and 0.481 +/- 0.118 (SE) MPa, respectively. In the first experiment, breakage occurred in 1 of 10 tendons in the control model at 27 hours, 2 of 10 in the NKISK model at 27 hours and 36 hours, 2 of 10 in the relaxin model both at 48 hours, and 0 of 10 in the mixture model. No breakage occurred in the second experiment at 27 hours.

Despite the higher stress in the control group of the first experiment, NKISK and relaxin treated tendons demonstrated significantly more creep than the controls from the same tail in both experiments. Figure 3 displays the induced creep in experimental models compared to that of the control model in the first experiment with all experimental values being statistically significant ($p<0.05$) from the 3 hour time point on.

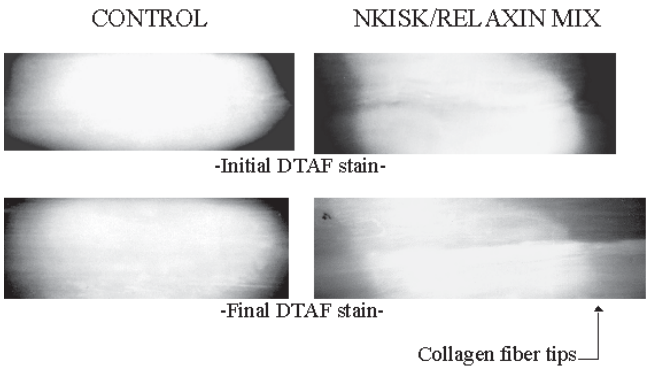

Figure 4. Representative initial (top row) and final (bottom row) photomicrographs of a DTAF mark in the control (left column) and NKISK/relaxin mixture (right column) groups. The final photomicrograph in the control model shows that the stained edges remain relatively sharp while in the NKISK/relaxin model, collagen fiber tips can be seen protruding from either side of the mark as would be expected with collagen fiber sliding.

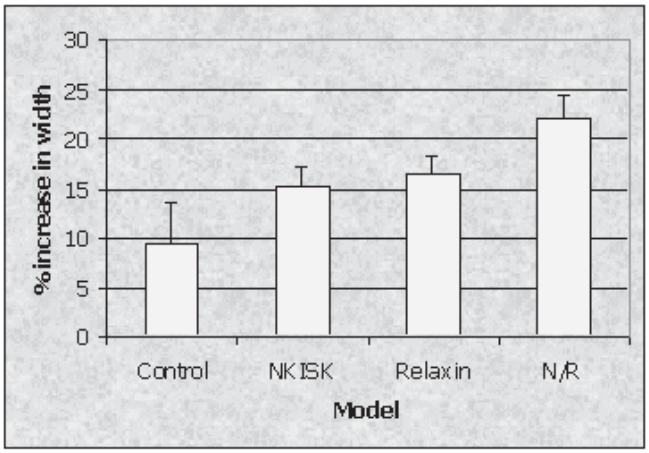

Figure 5. Percent increase in DTAF marking widths in the NKISK, relaxin, and mixture models all showed percent increases greater than in the control, but only the mixture model versus control was statistically significant ($p<0.05$). The hash marks represent the standard error.

Figure 4 shows representative initial and final photomicrographs of a DTAF mark in the control and NKISK/relaxin mixture groups. The final photomicrograph in the control model shows that the stained edges remain sharp while in the NKISK/relaxin model, it appears "fuzzy" and collagen fibers seem to be protruding from either side of the mark as would be expected with collagen fiber sliding.

Figure 5 shows that the DTAF marking widths in the NKISK, relaxin, and mixture models all showed percent increases greater than in the control though only the mixture model versus control was statistically significant.

DISCUSSION

NKISK is known to inhibit decorin-fibronectin binding[12] and postulated to interfere with interfibrillar bonding. Relaxin is known to allow stretching of collagenous tissues[1,8,13,18], but the mechanism is unknown. In this study, tendons exposed to NKISK and relaxin consistently stretched significantly more than the control tendons at each time interval. We feel that NKISK and relaxin potentiated tendon creep and that the changes in length are secondary to collagen fiber sliding as evidenced by DTAF photomicrography. As hypothesized, the fluorescence photomicrographs demonstrated stained collagen protruding from either side of the original mark as one would expect if fibers slid past one another and away from their initial location during changes in length. Actual fibril sliding could not be observed in this study with DTAF as collagen fibril diameters are 50-200nm and individual fibrils could not be resolved with light microscopy. We were able to observe translational collagen movement and it is possible that it may be at the fiber level rather than the fibril level.

DTAF selectively labels collagen in collagen-rich tissues such as tendon. By utilizing gel filtration experiments, Davison and Galbavy have shown that approximately 97% of applied DTAF dye will bind to collagen. The remaining small percentage binds to proteoglycan, glycoprotein and other stromal components. It is unlikely that the observed broadening of the bands is due to diffusion of the dye because of the covalent bonding of the DTAF to collagen. Likewise, it is unlikely that NKISK or relaxin affect the DTAF—collagen covalent bonding. Experiments have shown that collagen-rich cornea stained with concentric rings of DTAF in the growing rabbit show expansion of the stained ring, not diffusion from the ring, which would have narrowed the inner diameter[3].

The DTAF marking widths in the NKISK, relaxin, and mixture models all showed percent increases greater than in the control. Increased marking widths, in the absence of sliding, would require permanent fibril stretching, a phenomenon which is incompatible with the crosslinked structure of collagen fibrils. Collagen molecules wound into triple helices are cross-linked to one another in a staggered array to form fibrils, which by virtue of the fact that all connections are molecular, could be considered to be large “super molecules”. While perhaps not absolutely rigid, the tendons should at least be elastic and once unloaded for photomicrography, should return to their original lengths. The photomicrographs document collagen fibers protruding after unloading, thus stretching is unlikely to account for the observed changes.

Although this technique is limited to measuring movement only on the surface of the tendon, it seems unlikely that the collagen interactions are different in the depths of the tendon. NKISK and relaxin are small polypeptides and should penetrate the depths of the tendon quite readily. Interactions in the depths of tendon are likely similar to the surface because collagen composition is uniform throughout the tendon and it would be very unlikely to observe the increases in tendon length if the depth of the tendon’s length remained constant while the surface elongated.

All values were statistically significant in the first experiment, however, not in the second. The smaller number of tendons used (16 versus 40) in the second experiment was a result of the technically challenging and time consuming procedure involved in staining and photographing DTAF stained tendons. In the second experiment, there was statistical significance ($p<0.05$) only in the NKISK/relaxin model versus control for the percent increase in marking widths. In the NKISK and relaxin test solutions, the mean percent increase in marking widths were greater than the controls, however, the small “n”, or the large standard error, or both, made these differences not statistically significant.

We postulated that if NKISK and relaxin worked by different mechanisms they would have an additive effect when combined. No such additive effect was observed. Bundle ruptures were not likely the source of tendon elongation as this would have been indicated by sliding of large bundles in the DTAF photomicrographs.

There are now reports of three agents that interfere with fibril association (NKISK, relaxin and gentamycin) and to date, we are not aware of any reports that document these agents have anything in common. NKISK is known to inhibit decorin-fibronectin binding and postulated to interfere with interfibrillar bonding[12]. Relaxin is known to allow stretching of collagenous tissues, but the mechanism is unknown. Some authors propose that relaxin removes cross-linking of fibrils allowing them to slip and re-orient, which may be stimulated by increased collagenase expression and downregulation of collagen secretion[13,18]. Gentamycin is a small polycation and its size and charge configuration are very similar to the KKK tripeptide, which also allows increased tendon lengthening[11]. We hypothesize that it may also bind to the decorin-fibronectin binding site.

Though this study provides some understanding of vertebrate fibril sliding, much work remains to be done. Future work should include further investigations to elucidate the mechanisms by which collagen fibrils are presumably bound as well as the mechanism of collagen sliding. In this study, decorin-fibronectin bind-

ing appears to play an important role in the bonding of fibrils to one another to prevent constant sliding. These results provide further support for the "sliding fibril hypothesis" for length changes in collagenous tissues.

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